

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶: C12N 15/54, C12P 13/22, 1/00, 7/42, C12N 15/31, 1/21 // C12Q 1/48, (C12N 1/21, C12R 1:07, 1:13, 1:15, 1:185, 1:19, 1:425)	A1	(11) International Publication Number: WO 98/18937 (43) International Publication Date: 7 May 1998 (07.05.98)
(21) International Application Number: PCT/NL97/00583 (22) International Filing Date: 17 October 1997 (17.10.97) (30) Priority Data: 196 44 567.1 26 October 1996 (26.10.96) DE (71) Applicants (for all designated States except US): HOLLAND SWEETENER COMPANY V.O.F. [NL/NL]; Blekerij 52, NL-6212 XW Maastricht (NL). FORSCHUNGSZENTRUM JÜLICH GMBH [DE/DE]; D-52425 Jülich (DE). (72) Inventors; and (75) Inventors/Applicants (for US only): SPRENGER, Georg [DE/DE]; Schlossstrasse 4, D-52428 Jülich (DE). SIEWE, Ruth [DE/DE]; Gebhardtstrasse 10, D-76137 Karlsruhe (DE). SAHM, Hermann [DE/DE]; Wendelinusstrasse 71, D-52428 Jülich (DE). KARUTZ, Martin [DE/NL]; Bretagnestraat 35, NL-6137 JE Sittard (NL). SONKE, Theodorus [NL/NL]; Caeciliastraat 15, NL-6143 BK Sittard (NL). (74) Agent: DAUTZENBERG, Jozef, Marie, Andreas; Octrooibureau DSM, P.O. Box 9, NL-6160 MA Geleen (NL).		(81) Designated States: AL, AU, BA, BB, BG, BR, CA, CN, CU, CZ, EE, GE, HU, ID, IL, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, SL, TR, TT, UA, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: MICROBIAL PREPARATION OF SUBSTANCES FROM AROMATIC METABOLISM/II		
(57) Abstract <p>The invention makes available, by means of an increased provision of intracellular metabolic intermediates, in particular of phosphoenolpyruvate, alternative processes for the microbial preparation of substances, in particular of aromatic amino acids such as L-phenylalanine, in which processes the activity of a sugar-phosphorylating kinase is increased in a microorganism producing these substances. In preferred embodiments of the invention, the activity of a transport protein for the PEP-independent uptake of a sugar to be phosphorylated by the kinase, or the activity of a transaldolase and/or a transketolase is increased in addition. The invention also relates to gene structures, and to transformed cells carrying these gene structures, which make it possible to implement these processes in a particularly successful manner.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

- 1 -

MICROBIAL PREPARATION OF SUBSTANCES
FROM AROMATIC METABOLISM/II

5

The invention relates to a process for the microbial preparation of substances, in particular aromatic amino acids, in accordance with Claims 1-20, 36 and 37, gene structures in accordance with Claims 21-29, and transformed cells in accordance with Claims 30-35.

Microbially prepared substances, such as fine chemicals, in particular aromatic amino acids, are of great economic interest, with the requirement for amino acids, for example, continuing to increase. Thus, L-phenylalanine, for example, is used for preparing medicaments and, in particular, also in the preparation of the sweetener aspartame (α -L-aspartyl-L-phenylalanine methyl ester). L-tryptophan is required as a medicament and as an additive to animal feeds; there is likewise a need for L-tyrosine as a medicament and also as a raw material in the pharmaceutical industry. In addition to isolation from natural materials, biotechnological preparation is a very important method for obtaining amino acids in the desired optically active form under economically justifiable conditions. Biotechnological preparation is effected either using enzymes or using microorganisms. The latter, microbial, preparation enjoys the advantage that simple and inexpensive raw materials can be employed. Since the biosynthesis of amino acids in the cells is controlled in a wide variety of ways, a large number of attempts have already been made to increase product formation. Thus, amino acid analogs, for example, have been employed in order to switch off the regulation of biosynthesis. For example, mutants of *Escherichia coli* permitting an increased production of L-phenylalanine were obtained by

35

selecting for resistance to phenylalanine analogs (GB-2,053,906). A similar strategy also led to overproducing strains of *Corynebacterium* (JP-19037/1976 and JP-39517/1978) and *Bacillus* (EP-0,138,526). Furthermore, 5 microorganisms which have been constructed using recombinant DNA techniques are known in which the regulation of biosynthesis is likewise abolished, with the genes which encode key enzymes which are no longer subject to feedback inhibition being cloned and expressed. As a 10 prototype, EP-0,077,196 describes a process for producing aromatic amino acids in which a 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase (DAHP synthase) which is no longer subject to feedback inhibition is overexpressed in *E. coli*. EP-0,145,156 describes an *E.* 15 *coli* strain in which chorismate mutase/prephenate dehydratase are additionally overexpressed for the purpose of producing L-phenylalanine.

A feature common to the abovementioned strategies is that the intervention for improving 20 production is restricted to the biosynthesis pathway which is specific for the aromatic amino acids. However, in order to increase production still further, efforts must be made to improve the provision of the primary metabolites, phosphoenolpyruvate (PEP) and erythrose-4-phosphate (Ery4P), which are required for producing 25 aromatic amino acids.

PEP is an activated precursor of the glycolysis product pyruvate; Ery4P is an intermediate in the pentose phosphate pathway.

30 Several strategies have been described in the literature for increasing the availability of PEP, for example by decreasing the activity of PEP carboxylase (Miller J.E. et al., J. Ind. Microbiol. 2 (1987) 143-9; EP-0,140,606) or by increasing the activity of PEP

synthase (Chao Y.P. et al., J. Biol. Chem. 269 (1994) 5122-26; Patniak R. et al., Biotechnol. Bioeng. 46 (1995) 361-70). It has furthermore been found that, in thiamine-auxotrophic and lipoic acid-auxotrophic mutants of

5 *Enterobacter aerogenes*, the activity of the pyruvate dehydrogenase complex is reduced, as a result of which the outflow of PEP via pyruvate to acetyl-CoA and into the citric acid cycle is decreased, with tryptophan being formed as a consequence (Oita S. et al., J. Ferm. Bioeng.

10 69 (1990) 256-8). In addition, microorganisms which take up glucose by way of a PEP:sugar phosphotransferase system (PTS) (*pts*⁻ strains) consume equimolar quantities of PEP for this transport process with the result that this PEP is no longer available for synthesizing aromatic compounds

15 (Postma P.W. et al., Microbiol. Rev. 57 (1993) 543-594).

It has recently been demonstrated (Flores N. et al., Nature Biotechnol. 14 (1996) 620-3) that a spontaneous glucose-positive revertant of a PTS-negative (*pts*⁻) mutant of *Escherichia coli* funnelled glucose into

20 the cells by way of the GalP system and was capable of growing on glucose. An increased formation of the intermediate DAHP was observed in this study when the transketolase gene *tktA* was expressed in addition. Frost and Draths take into consideration the possibility of improving the provision of PEP by inactivating the PTS com-

25 pletely and subsequently overexpressing the *glf* and *glk* genes from *Zymomonas mobilis* (Frost and Draths, Ann. Rev. Microbiol. 49 (1995) 557-79). However, it is not possible to draw any conclusion from this paper about the

30 efficiency of such an intervention just as it is not possible to assess any consequences which such an intervention might have for the production of aromatic intermediates in *pts*⁻ strains. In particular, therefore, it cannot be derived that the mere overexpression of or

increase in the activity of a sugar-phosphorylating kinase in a microorganism has a favourable effect on its substance-producing performance. Rather, as appears from J. Bacteriol. 122 (1975), 1189-1199 (Curtis et al.; see
5 below), a person skilled in the art would reject such an intervention because, as this reference shows, the sugar-phosphorylating kinases obviously play no role in the production of substances.

The object of the invention is, therefore, to
10 make available an alternative process for producing substances, in particular aromatic amino acids, which process is characterized by an increased provision of PEP for the synthesis of these substances.

Surprisingly, the object is achieved, in accor-
15 dance with the invention, by making available a process for the microbial preparation of substances, in which process the activity of a sugar-phosphorylating kinase is increased in a microorganism which produces these substances. This result is particularly surprising since it
20 is in no way self-evident that mere overexpression of a sugar-phosphorylating kinase is of importance in the provision of PEP and consequently of importance for producing substances.

When bacterial cells (e.g. *Escherichia coli*)
25 grow on hexoses, such as glucose or fructose, under natural conditions, the greater part of these substrates is taken up by the bacterial cells by way of PEP:hexose phosphotransferase systems and phosphorylated, with PEP being consumed. The effect of sugar-phosphorylating
30 kinases is therefore restricted to activating those sugars which are present in the cell in a non-phosphorylated state because they were taken up by means of a PEP-independent transport system. Sugars of this nature can result, for example, from reactions in which intracellular

disaccharides and oligosaccharides, such as trehalose, lactose, maltose or maltodextrin, are hydrolyzed.

For example, glucokinase has a subordinate function in *Escherichia coli* which is growing on glucose
5 (Curtis and Epstein, J. Bacteriol. 122, (1975), 1189-1199). It does not exert any direct influence on substance fluxes, either by way of glycolysis for providing PEP or by way of the pentose phosphate pathway for providing Ery4P. Therefore, a person skilled in the art would not
10 assume that in microorganisms phosphorylating kinases play a role with regard to substance-producing capability.

Consequently, the effect, which is described within the context of this invention, of the overexpression of a sugar-phosphorylating kinase on the
15 production of substances, in particular aromatic amino acids, is completely unexpected.

The inventors assume that overexpression of the kinase leads to an increase in the proportion of intracellular sugar phosphates whose activation takes
20 place on the basis of ATP functioning as the energy donor. As a result, the quantity of PEP employed for this purpose decreases, something which is beneficial for the synthesis of secondary products of PEP. Despite many years of detailed studies on the preparation of substances from
25 aromatic metabolism, simply increasing the activity of a kinase has never hitherto been postulated in the state of the art.

Within the meaning of the invention, substances are to be understood as being, for example, fine chemicals
30 such as aromatic amino acids, indigo, indoleacetic acid, adipic acid, melanin, quinones and benzoic acid, as well as their potential derivatives and secondary products - or, generally, derivatives of intermediates of the pentose phosphate pathway. Within the context of this invention,

all these substances are also regarded as being substances from aromatic metabolism. In this connection, it may be noted that other genetic alterations to the microorganisms producing the substances are required, in addition to the novel interventions, in order to prepare indigo, adipic acid and other unnatural secondary products.

The process for the microbial preparation of substances is therefore particularly advantageous when substances in whose synthesis PEP is involved are being prepared.

When a sugar-phosphorylating kinase is being employed, it is advisable to use a hexose-phosphorylating kinase, preferably a kinase from *Zymomonas mobilis*, in particular glucokinase (Glk) from *Zymomonas mobilis*. When the latter is used, the protein-encoding gene, *glk*, is derived, for example, from *Z. mobilis* ATCC 10988, ATCC 29191 or ATCC 31821. Other genes for hexose-phosphorylating kinases from bacteria whose gene products phosphorylate hexoses while consuming ATP, such as a fructokinase or a galactokinase, are likewise suitable for the novel process. Furthermore, genes for, for example, kinases from eukaryotic microorganisms, such as *Saccharomyces cerevisiae*, or, in a general manner, genes for sugar-phosphorylating kinases from other organisms, are also suitable, provided they can be expressed in a functional manner in the microorganisms, in particular amino acid-producing microorganisms (amino acid producers) and can operate without using PEP to phosphorylate the sugars. It is particularly advisable for it to be possible to express the sugar-phosphorylating kinases in amino acid producers. The glucokinase gene *glk*, for phosphorylating glucose, which gene is isolated from *Z. mobilis* ATCC 29191, is particularly suitable for preparing aromatic amino acids in accordance with the novel process.

Overexpressing the sugar-phosphorylating kinase in accordance with the present invention makes available an alternative process which decreases the consumption of PEP for activating sugars in the cell by increasing the use of alternative energy donors, such as ATP as the energy donor. As a result, an increased quantity of PEP is available for the microbial synthesis of substances in whose synthesis PEP is involved.

In a preferred embodiment of the invention, the activity of a transport protein for the PEP-independent uptake of a sugar which is to be phosphorylated by a kinase is increased in addition to increasing the activity of the sugar-phosphorylating kinase.

This embodiment also includes increasing the activity of a transport protein for the PEP-independent uptake of such a sugar in a substance-producing microorganism which is able to take up the relevant sugar by means of a PEP-dependent transport system. The additional integration of a PEP-independent transport system makes it possible to increase the provision of non-phosphorylated sugars in the substance-producing microorganism. These sugars can be converted into activated sugar phosphates by the kinases which are in each case relevant, with consumption of ATP, and then subjected to further metabolism. PEP is not required as an energy donor for these reactions and is consequently available in increased quantity, assuming a constant metabolic flux in glycolysis and the pentose phosphate pathway, for condensing with Ery4P to form the primary metabolite in the general pathway for biosynthesizing aromatic compounds, i.e. deoxy-D-arabinoheptulosonate-7-phosphate (DAHP), and consequently for producing substances such as aromatic compounds.

In the case of the transport protein, activity

is to be understood, in this context, as being the rate of protein-mediated uptake. In order to increase the activity of the PEP-independent transport protein in PTS-negative strains, it is particularly advantageous, in contrast to the two-step procedure already described for the *Zymomonas mobilis* *glf* gene, that the PTS can be eliminated and the gene for the transport protein can be introduced in one step. According to the invention, a strategy of this nature can be implemented by inserting the gene, for example, into a gene of the *ptsHI-crr* operon, e.g. into the *ptsI* gene or into another *pts* locus, and makes it easier to obtain the desired mutants. This approach completely abolishes vectorial sugar transport through the cell membrane and the PEP-dependent process for phosphorylating sugars which is coupled to it. At the same time, other functions of the PTS can be preserved.

Inserting the gene for the transport protein downstream of a PTS promoter is also advantageous since there is then no need to introduce a separate gene structure and the expression of the protein does not, due to the natural level of expression, destabilize the recombinant cell.

It is advisable to use a facilitator as the transport protein for the PEP-independent uptake of a sugar, that is a transport protein which acts in accordance with the principle of protein-mediated facilitated diffusion. In particular, it is suitable to use the glucose facilitator protein (Glf) from *Zymomonas mobilis*. When the latter is used, the protein-encoding gene, *glf*, is obtained, for example, from *Z. mobilis* ATCC 10988, ATCC 29191 or ATCC 31821. However, other bacterial sugar transport genes whose gene products transport glucose, fructose or sucrose, for example, and in doing so do not use any PEP, for example the GalP system from *Escherichia*

coli, are also suitable for the novel process. Genes for sugar transport systems, such as HXT1 to HXT7, from eukaryotic microorganisms, such as *Saccharomyces cerevisiae*, *Pichia stipitis* or *Kluyveromyces lactis*, or
5 sugar transport genes from other organisms in general, can also be used, provided that they can be expressed in a functional manner in the microorganisms and that the gene products can, in this context, operate without using PEP for phosphorylation and/or for transporting the sugars. It
10 is particularly advisable for it to be possible to express the sugar transport genes in amino acid producers.

It is particularly suitable, therefore, to use the facilitator gene *glf*, isolated from *Z. mobilis* ATCC 31821, for taking up sugars such as glucose, fructose or
15 mannose when preparing aromatic amino acids in accordance with the novel process. (Parker C. et al., Mol. Microbiol. 15 (1995) 795-802; Weisser P. et al., J. Bacteriol. 177 (1995) 3351-4). The *glf* gene, in particular, should preferably be inserted at a low gene copy number in order
20 to avoid harmful effects on the cell due to excessive expression of membrane proteins. Thus, a gene copy number of from 2 to 5 is preferred, for example, for the *glf* gene. It is particularly advantageous, as already mentioned above, for the *glf* gene to be inserted into one
25 of the genes of the *ptsHI-crr* operon.

In a particularly preferred embodiment, the activity of a transaldolase and/or the activity of a transketolase is increased in addition to increasing the activity of a sugar-phosphorylating kinase or to increas-
30 ing the activity of a sugar-phosphorylating kinase and of a PEP-independent transport protein.

The additional increase in the activity of one of these proteins (that is, the transaldolase and the transketolase), or of both the proteins, makes it possible

to achieve an even higher production of substances, in particular aromatic amino acids, due to the fact that Ery4P is provided in increased quantity for the condensation with PEP to form the primary metabolite in the general pathway for biosynthesizing aromatic compounds, i.e. deoxy-D-arabinoheptulosonate-7-phosphate (DAHP).

In regard to increasing the activity of a transaldolase, preference is given to increasing the activity of a transaldolase from *Escherichia coli* and, in particular, to increasing the activity of *Escherichia coli* transaldolase B (TalB). When the corresponding talB gene is used, this gene preferably originates from *Escherichia coli* K12 or from a strain derived therefrom. However, besides this, any other gene is also suitable whose gene product catalyzes a reaction which corresponds to that of transaldolase, that is the conversion of sedoheptulose-7-phosphate plus glyceraldehyde-3-phosphate to Ery4P and fructose-6-phosphate.

With regard to increasing the activity of a transketolase, preference is given to increasing the activity of a transketolase from *Escherichia coli* and, in particular, the activity of *Escherichia coli* transketolase A (TktA). When the corresponding tktA gene is used, this gene preferably originates from *Escherichia coli* K12 or from a strain which is derived therefrom. However, besides this, any other gene is also suitable whose gene product catalyzes a reaction which corresponds to that of transketolase, that is the conversion of ribose-5-phosphate plus xylulose-5-phosphate to sedoheptulose-7-phosphate plus glyceraldehyde-3-phosphate or to the conversion of xylulose-5-phosphate plus Ery4P to fructose-6-phosphate plus glyceraldehyde-3-phosphate.

The availability of PEP for producing the first intermediate of aromatic amino acid metabolism can be

limited in microorganisms in which the material flow towards Ery4P is increased. In these cases, it can be advantageous to decrease or abolish other metabolic reactions which consume PEP, for example the reaction of the PEP:sugar phosphotransferase system (PTS), which catalyzes a PEP-dependent sugar uptake, provided such reactions are present.

According to the invention, use can be made both of organisms which exhibit the natural level of PTS activity and also, in order to improve the process still further, of PTS mutants in which the activity of the PTS is decreased. Within the meaning of this invention, "decrease" means that the activity has been lowered down to a residual activity of 1% of the natural activity. A decrease of this nature can be effected either at the enzymic level or by using genetic methods, for example by using alternative, highly repressible promoters for expressing the *pts* genes or by inserting a *glf* gene and/or a *glk* gene into the chromosome and, in particular, into the locus of the *ptsI* gene, a procedure which simultaneously stabilizes the recombinant DNA in the chromosome (segregation stability) and consequently means that the use of a vector can be dispensed with. Furthermore, when a regulatable promoter is used, influence can also be exerted on the activity of the PTS, during culture, by adding inducers or inhibitors of the corresponding promoter.

Within the meaning of the invention, measures for increasing the activity are to be understood as being all measures which are suitable for increasing the activity of the kinase, of the transport protein, of the transaldolase and of the transketolase. The following measures are particularly suitable for this purpose:

- introduction of genes, for example using vectors or

temperate phages;

- increasing the gene copy number, for example using plasmids with the aim of introducing the genes according to the invention into the microorganism at an increased copy number, that is at a copy number which is slightly (e.g. from 2 to 5 times) increased to highly (e.g. from 15 to 50 times) increased;
- increasing gene expression, for example by increasing the rate of transcription, for example by using promoter elements such as Ptac, Ptet or other regulatory nucleotide sequences, and/or by increasing the rate of translation, for example by using a consensus ribosome binding site;
- increasing the endogenous activity of enzymes which are present, for example by means of mutations which are produced in a random manner by conventional methods, for example using UV irradiation or mutation-eliciting chemicals, or by means of mutations, such as deletion(s), insertion(s) and/or nucleotide exchange(s), which are produced in a specific manner using genetic engineering methods;
- increasing the activity of enzymes by altering the structure of enzymes, for example by means of mutagenesis using physical, chemical or molecular biological or other microbiological methods;
- using deregulated enzymes, for example enzymes which are no longer subject to feedback inhibition;
- introduction of corresponding genes which encode the deregulated enzymes.

Combinations of the abovementioned methods and other, analogous methods can also be employed for increasing the activity. The endogeneous activity of transport proteins can be increased, for example, by cloning the gene using the abovementioned methods, for example, or by selecting mutants which exhibit an

increased transport of substrates.

Preferably, the activity is increased by integrating the gene, or the genes, into a gene structure, or into several gene structures, with the gene or the genes
5 being introduced into the gene structure as (a) single copy(ies) or at an increased copy number.

Within the meaning of the invention, a gene structure is to be understood as being a gene and any nucleotide sequence which carries the genes according to
10 the invention. Appropriate nucleotide sequences can, for example be plasmids, vectors, chromosomes, phages or other nucleotide sequences which are not closed circularly.

A chromosome within the meaning of the invention is a chromosome into which at least one gene according to
15 the invention has been inserted, with the resulting nucleic acid sequence containing at least one gene, or one gene copy, more than it naturally contained in this chromosome. Thus, for example, homologous recombination within a gene locus leads to a chromosome which does not
20 necessarily have to differ from the natural form. The chromosomes which are prepared by homologous recombination are not, therefore, to be regarded as being in accordance with the invention if the natural number of homologous genes is not exceeded.

25 Within the meaning of the invention, a gene structure is also to be understood as being a combination of the abovementioned gene carriers, such as vectors, chromosomes and temperate phages, on which the genes according to the invention are distributed. For example,
30 two *glk* genes can be introduced into the cell on a vector or two *glk* genes can be inserted into a chromosome. In addition, a further gene can, for example, be introduced into the cell using a phage. The same applies to the other genes according to the invention. These examples are not

intended to exclude other combinations of gene distributions from the invention. In any case, it is crucial that the number of genes contained in the microorganism exceeds the natural number of the corresponding genes.

Preferably, the number of *glf* genes, for example, per gene having the same effect will be increased by a factor of from 2 to 5 in order to achieve the increase in activity according to the invention. No cell-toxic effect will appear at these concentrations. However, it is also conceivable to introduce the genes according to the invention into the microorganism at a higher copy number of up to 50 gene copies of a form having the same effect.

In the novel process for producing substances, preference is given to employing microorganisms in which one or more enzymes which are additionally involved in synthesizing the substances are deregulated and/or have an increased activity.

These enzymes are, in particular, the enzymes of aromatic amino acid metabolism and, especially, DAHP synthase, shikimate kinase and chorismate mutase/prephenate dehydratase, and also all the other enzymes which are involved in synthesizing intermediates of aromatic metabolism and their secondary products.

Apart from the enzymes according to the invention, the deregulation and overexpression of DAHP synthase in particular is of importance for preparing substances such as adipic acid, bile acid and quinone compounds and their derivatives. In addition, shikimate kinase should be deregulated, and its activity increased, in order to achieve superelevated synthesis of, for example, L-tryptophan, L-tyrosine, indigo and derivatives of hydroxybenzoic acid and aminobenzoic acid and naphtho-

quinones and anthroquinones and also their secondary products. Deregulated and overexpressed chorismate mutase/prephenate dehydratase is additionally of particular importance for efficiently producing phenylalanine and phenylpyruvic acid and their derivatives. However, this is also intended to encompass all the other enzymes whose activities contribute to the biochemical synthesis of substances, that is compounds whose production is promoted by the provision of PEP, for example CMP-ketodeoxyoctulosonic acid, UDP-N-acetylmuramic acid, N-acetylneuraminic acid or chorismic acid. In this context, the increased provision of PEP can not only have a positive effect on the synthesis of DAHP but can also favour the insertion of a pyruvate group in the synthesis of 3-enolpyruvylshikimate-5-phosphate as a precursor of chorismate.

It may be noted that further genetic alterations to the substance-producing microorganisms, in addition to the novel interventions, are required for the purpose of preparing indigo, adipic acid and other unnatural secondary products.

The novel process is suitable for preparing aromatic amino acids, in particular L-phenylalanine. In the latter case, the gene expression and/or the enzyme activity of a deregulated DAHP synthase (e.g. in *E. coli* AroF or AroH) and/or of a likewise deregulated chorismate mutase/prephenate dehydratase (PheA) is preferably increased.

Escherichia species, and also microorganisms of the genera *Serratia*, *Bacillus*, *Corynebacterium* or *Brevibacterium*, and other strains which are known from conventional amino acid methods, are suitable for use as production organisms. *Escherichia coli* is particularly suitable.

A further object of the invention is to provide suitable gene structures, and transformed cells carrying these gene structures, which enable the process to be implemented in a particularly successful manner.

5 Within the context of the invention, novel gene structures are now, firstly, made available which contain, in recombinant form, a gene encoding a sugar-phosphorylating kinase and a gene for a transport protein for the PEP-independent uptake of a sugar, with the
10 exception of the combination of the *Zymomonas mobilis* genes for the *glk* kinase and the *glf* transport protein. The exception of the specific combination of the *Zymomonas mobilis glk* and *glf* genes only is based on the fact that a gene structure of this nature has already been frequently
15 described in the literature. The background of these studies was the investigation of the ability of the *Zymomonas mobilis glf* and *glk* genes to be expressed in *Escherichia coli* and the possibility of in this way enabling PTS⁻ mutants to grow on PTS sugars (Snoep et al.,
20 J. Bacteriol. 176 (1994) 2133-35). However, an effect of Glk, in particular, on the preparation of substances according to the invention is not likely, or to be expected, on the basis of those results. The novel gene structures are therefore unexpectedly effective for
25 preparing substances and are, in particular, also effective in PTS⁺ strains.

 Within the context of the invention, novel gene structures are secondly made available which contain, in recombinant form, a) a gene encoding a sugar-phosphorylating kinase or genes encoding a sugar-phosphorylating
30 kinase and a transport protein for the PEP-independent uptake of a relevant sugar, and b) at least one gene encoding a transaldolase or a transketolase. In these first and second gene structures, it is preferred that the

gene for the kinase encodes a hexose-phosphorylating kinase and the gene for the transport protein encodes a facilitator.

5 The genes for the kinase and for the transport protein are derived, in particular, from *Zymomonas mobilis*. The genes for the transaldolase and for the transketolase, mentioned in the second gene structures, are derived, in particular, from *Escherichia coli*. Gene structures are particularly advantageous in which the gene
10 for the kinase is *Zymomonas mobilis glk* and the gene for the transport protein is *Zymomonas mobilis glf* and in which, where appropriate, the gene for transaldolase is *Escherichia coli talB* and the gene for transketolase is *Escherichia coli tktA*.

15 The appropriate genes are isolated, and the cells are transformed, in accordance with current methods: when cloning the *Zymomonas mobilis glk* glucokinase gene or the *Zymomonas mobilis glf* transport gene, for example, the polymerase chain reaction (PCR) method, for example, for
20 specifically amplifying the gene using chromosomal DNA from *Zymomonas mobilis* strains ATCC 29191 or ATCC 31821 is suitable, as is also the heterologous complementation of *Escherichia coli* mutants which are defective in PTS functions and which therefore are unable to transport
25 glucose, for example (Snoep J.L. et al., J. Bacteriol. 174 (1994) 1707-8; Parker C. et al., Mol. Microbiol. 15 (1995) 795-82; Weisser P. et al., J. Bacteriol. 177 (1995) 3351-4). After isolating the genes and recombining them in vitro with known low copy number vectors such as pACYC184,
30 pACYC177, pSC101 or pZY507 (Weisser P. et al., J. Bacteriol. 177 (1995) 3351-4), the host cell is transformed using chemical methods, electroporation, transduction or conjugation.

The complete nucleotide sequences of the *talB*

and *tktA* genes from *Escherichia coli* K12 are known (Yura T. et al., Nucl. Acid Res., 20 (1992) 3305-8; Sprenger G.A., Biochim. Biophys. 1216 (1993) 307-10; Sprenger G.A. et al., J. Bacteriol. 177 (1995) 5930-9) and deposited in
5 databases such as that of the EMBL in Heidelberg. When the *Escherichia coli talB* gene is being cloned, PCR is suitable for specifically amplifying the gene using chromosomal DNA from *Escherichia coli* K12 strains (Sprenger G.A. et al., J. Bacteriol. 177 (1995) 5930-9).
10 The homologous complementation of a transketolase-deficient mutant is suitable, for example, when cloning the *Escherichia coli tktA* gene (Sprenger G.A. in: Bisswanger H. et al., Biochemistry and physiology of thiamine diphosphate enzymes, VCH (1991) 322-6).
15 The isolated kinase gene can be integrated, together with one or more of the genes described within the context of the invention, in any combination, into a gene structure or into several gene structures. Without considering the precise allocation to gene structures,
20 this leads to combinations such as *glk*, *glk + talB*, *glk + tktA*, *glk + glf + talB*, *glk + glf + tktA*, *glk + talB + tktA* or *glk + glf + talB + tktA*. When the genes are allocated, *glf* is preferably introduced at low copy number into the gene structure or the gene structures in order to
25 avoid the possible negative effects of the overexpression of a membrane protein.

Gene structures are advantageous which contain at least one regulatory gene sequence which is assigned to one of the genes. Thus, reinforcement of regulatory
30 elements can preferably be effected at the level of transcription by, in particular, reinforcing the transcription signals. This can be effected, for example, by increasing the activity of the promoter or the promoters by altering the promoter sequences which are

located upstream of the structural genes or by completely replacing the promoters with more effective promoters. Transcription can also be reinforced by exerting an appropriate influence on a regulatory gene which is
5 assigned to the genes; in addition to this, however, it is also possible to reinforce translation by, for example, improving the stability of the messenger RNA (mRNA).

The most suitable gene structures are those in which at least one of the described genes is incorporated
10 such that it is under the control of an inducible promoter.

When the genes are arranged on a gene structure according to the invention, a promoter can be located upstream of a gene or be located, as a common promoter,
15 upstream of several genes, or use can be made of two opposed promoters between which the genes are arranged such that they are read off in opposite directions. In this context, the *glf* gene, for example, can be located downstream of a relatively weak promoter (e.g. *Ptet*) and
20 other genes can be under the control of the *tac* promoter. One or more DNA sequences can be located upstream and/or downstream of the genes contained in a gene structure, with or without an upstream promoter or with or without an assigned regulatory gene. By means of using inducible
25 promoter elements, e.g. *lacI^q/Ptac*, it is possible to switch on new functions (induction of enzyme synthesis), for example by adding chemical inducers such as isopropylthiogalactoside (IPTG).

The object of the invention is also achieved by
30 providing transformed cells which harbour a gene structure according to the invention in replicable form.

Within the meaning of the invention, a transformed cell is to be understood as being any microorganism which carries a gene structure according to

the invention, which gene structure brings about the increased formation of substances in the cell.

The host cells can be transformed by means of chemical methods (Hanahan D., J. Mol. Biol. 166 (1983) 557-580) and
5 also by means of electroporation, conjugation or transduction.

For the transformation, it is advantageous to employ host cells in which one or more enzymes which are additionally involved in the synthesis of substances are
10 deregulated and/or have an increased activity. A microorganism strain, in particular *Escherichia coli*, which is producing an aromatic amino acid or another substance according to the invention is transformed with the gene structure which contains the relevant genes. For
15 transforming with the gene structures, it is advantageous to employ host cells in which the activity of the PEP-dependent sugar uptake system, if present, is decreased or abolished.

In particular, transformed cells are provided
20 which are able to produce an aromatic amino acid, with the aromatic amino acid preferably being L-phenylalanine.

Using the novel process, and the microorganism which has been transformed in accordance with the teaching of the invention, a broad spectrum of substrates can be
25 employed for producing substances. Within the context of the invention, a process for the microbial preparation of substances is consequently also provided in which cells which have been transformed in accordance with the invention and in which a gene structure is present which
30 contains at least one regulatory gene sequence which is assigned to one of the genes are cultured, with enzyme synthesis being induced in the microorganisms after at least 2 cell divisions (beginning of the exponential growth phase). Consequently, the production of the micro-

organisms can be increased independently of their growth.

In a particularly preferred embodiment of the novel process, transformed cells are employed which, in addition to PEP, also contain an increased availability of other metabolites of central metabolism. These metabolites include, for example, α -oxoglutarate or oxaloacetate, which result from intracellular synthetic processes, or else are made available to the growing cells by feeding the corresponding substances, or their precursors, such as fumarate or malate, as metabolites of the citric acid cycle.

The following strains have been deposited in the DSMZ under the terms of the Budapest Treaty:

- DSMZ 11208 *Escherichia coli* AT2471/pZY507glk
- DSMZ 11207 *Escherichia coli* AT2471/pZY507glfglk
- DSMZ 11206 *Escherichia coli* AT2471GP704glfint PTS⁻
- DSMZ 11205 *Escherichia coli* AT2471glfint PTS⁻

The host organism employed, i.e. AT2471, has been deposited by Taylor and Trotter (Bacteriol. Rev. 13 (1967) 332-53) in the CGSC under number 4510 and is freely accessible.

The text which follows is intended to indicate the materials and methods employed and to support the invention with experimental examples and comparative examples:

General methods

In the genetic studies, strains of *E. coli* were, unless otherwise indicated, cultured on LB medium consisting of Difco bacto-tryptone (10 g.l⁻¹), Difco yeast extract (5 g.l⁻¹) and NaCl (10 g.l⁻¹). Depending on the resistance properties of the strains employed,

carbenicillin ($20-100 \text{ mg} \cdot \text{l}^{-1}$) and/or chloramphenicol ($17-34 \text{ mg} \cdot \text{l}^{-1}$) was/were added to the medium if necessary. For this, carbenicillin was first of all dissolved in water, and chloramphenicol in ethanol, and the solutions were
5 added, after having been sterilized by filtration, to the previously autoclaved medium. Difco bacto-agar (1.5%) was added to the LB medium for preparing agar plates.

Plasmid DNA was isolated from *E. coli* by means of alkaline lysis using a commercially available system
10 (Quiagen, Hilden). Chromosomal DNA was isolated from *E. coli* and *Z. mobilis* as described by Chen and Kuo (Nucl. Acid Res. 21 (1993) 2260).

Restriction enzymes, DNA polymerase I, alkaline phosphatase, RNase and T4 DNA ligase were used in accordance with the producers' instructions (Boehringer,
15 Mannheim, Germany or Promega, Heidelberg, Germany). For restriction analysis, the DNA fragments were fractionated in agarose gels (0.8%) and isolated from the agarose by means of extraction using a commercially available system
20 (Jetsoorb Genomed, Bad Oeynhausen, Germany).

For Southern analyses, chromosomal DNA ($10 \mu\text{g}$) was digested with restriction enzymes, size-fractionated by gel electrophoresis and transferred to a nylon membrane (Nytran 13, Schleicher and Schuell, Dassel, Germany) by
25 means of vacuum-mediated diffusion (VacuGene System, Pharmacia, Feiburg, Germany). Appropriate DNA fragments were isolated, labelled with dioxigenin-dUTP and used as probes. Labelling, hybridization, washing procedures and detection were performed with the aid of a commercially
30 available labelling and detection system (Boehringer, Mannheim, Germany).

For transformation, the cells were incubated at 37°C and 200 rpm for 2.5-3 h in LB medium (5 ml tubes). At an optical density (620 nm) of approx. 0.4, the cells were

centrifuged down and taken up in one tenth the volume of TSS (LB medium containing 10% (w/v) PEG 8000, 5% (v/v) DMSO and 50 mM MgCl₂). After a 30-minute incubation at 4°C with from 0.1 to 100 ng of DNA, and subsequent incubation
5 at 37°C for 1 h, the cells were plated out on LB medium containing an appropriate antibiotic.

Example 1

Preparation of pZY507glk and pZY507glfglk as prototypes of 10 plasmid-based gene structures according to the invention

Plasmid pZY507 (Weisser et al. 1995 J. Bacteriol 177: 3351-3345) was opened using the restriction enzymes *Bam*HI and *Hind*III and the larger fragment (10.1 kB) was isolated. The *glk* and *glf* genes were obtained and
15 amplified, as described by Weisser et al. (J. Bacteriol. 177 (1995) 3351-3354), using PCR (Mullis K.B. et al., Meth. Enzymol. 155 (1987) 335-50). The chromosomal DNA of *Zymomonas mobilis* was used for amplifying the *glk* gene. In this case, the choice of primers was used to insert
20 additional cleavage sites (*Kpn*I and *Hind*III). The *glf* gene was amplified using plasmid pZY600 (Weisser et al. 1995 J. Bacteriol 177: 3351-3345) as a template. In this case, the choice of primers was used to introduce a *Bam*HI cleavage site and a *Kpn*I cleavage site. The genes were then
25 introduced in various combinations into vector pZY507 using these unique cleavage sites. The recombinant plasmids pZY507glk, pZY507glf and pZY507glfglk were obtained after transforming *E. coli* and cloning the transformants. These vectors confer resistance to
30 chloramphenicol, contain the *lacI*^q-*tac* promoter system and are of low copy number.

The resulting transformants were stored at -80°C on LB medium in the form of glycerol cultures (30%). When needed, the glycerol cultures were thawed directly before

use.

Example 2

Determination of the enzyme activity of a glucokinase

- 5 In order to determine the activity of the glucokinase, the cells of *E. coli* AT2471 and of the corresponding plasmid pZY507glfglk-harboursing mutant AT2471/pZY507glfglk were cultured in mineral medium. This consisted of sodium citrate·3H₂O (1.0 g·l⁻¹), MgSO₄·7H₂O
10 (0.3 g·l⁻¹), KH₂PO₄ (3.0 g·l⁻¹), K₂HPO₄ (12.0 g·l⁻¹), NaCl (0.1 g·l⁻¹), (NH₄)₂SO₄ (5.0 g·l⁻¹), CaCl₂·2H₂O (15.0 mg·l⁻¹), FeSO₄·7H₂O (0.75 g·l⁻¹), and L-tyrosine (0.04 g·l⁻¹). Additional minerals were added in the form of a trace element solution (1 ml·l⁻¹), which was composed of
15 Al₂(SO₄)₃·18H₂O (2.0 g·l⁻¹), CoSO₄·6H₂O (0.7 g·l⁻¹), CuSO₄·5H₂O (2.5 g·l⁻¹), H₃BO₃ (0.5 mg·l⁻¹), MnCl₂·4H₂O (20.0 g·l⁻¹), Na₂MoO₄·2H₂O (3.0 g·l⁻¹), NiSO₄·3H₂O (2.0 g·l⁻¹) and ZnSO₄·7H₂O (15.0 g·l⁻¹). Vitamin B1 (5.0 mg·l⁻¹) was dissolved in water and added, having been sterilized by
20 filtration, to the medium after the latter had been autoclaved, as were carbenicillin and/or carbenicillin and chloramphenicol as the need arose. Glucose (30 g·l⁻¹) was autoclaved separately and likewise added to the medium after the latter had been autoclaved.
- 25 For the experiment, shaking flasks (1000 ml containing 100 ml of mineral medium) were inoculated with 2 ml of glycerol culture and incubated at 37°C and 150 rpm for 72 h on an orbital shaker. After about 7 divisions, and after reaching an optical density (620 nm) of ≈ 1, the
30 cells were induced by adding 15-100 μM IPTG. Parallel experimental mixtures were set up for checking the induction of the cells, one of which mixtures was induced by adding IPTG (20 μM). 20 ml of culture broth were removed from all the mixtures both directly before adding

the inducer to the appropriate flasks and at 1 and 3 h after the time of induction, and the cells were sedimented at 6000 g for 10 min and at 4°C.

The harvested cells were washed in 100 mM tris/HCl buffer (pH 8.0) containing 1.2 mM ATP and 11.2 mM MgCl₂. The cells in the sediment were disrupted by ultrasonic treatment (Branson 250 Sonifier fitted with a microtip) in a sonication cycle of 25% and at an intensity of 40 watts for 4 min per ml of cell suspension. Following centrifugation at 18000 g for 30 min at 4°C, the supernatant (crude extract) was used for measuring the activity of the glucokinase.

The activity of the glucokinase in the crude extract was determined using the method described by Doelle et al. (Eur. J. Appl. Microbiol. 14 (1982) 241-246). The turnover of 1 µmol of NADH per min was defined as 1 U.

The protein concentration in the crude extract was determined as described by Bradford M.M. (Anal. Biochem. 72 (1976) 248-254) using a commercially obtainable colour reagent. Bovine serum albumin was used as the standard.

Table 2 shows the results of the enzyme measurements when using the host strain *E. coli* 2471 and its mutant *E. coli* 2471/pZY507glkglf. Both strains were found to have a glucokinase activity of about 23 mU·(mg of protein)⁻¹ at the time of induction. While this activity only increased to 36 mU·(mg of protein)⁻¹ after a further three hours of culture in the case of the host strain, the glucokinase activity of the strain harbouring plasmid pZY507glfglk was found to increase to 112 mU·(mg of protein)⁻¹. It was possible to increase the activity of the glucokinase still further to 657 mU·(mg of protein)⁻¹ by inducing the cells which were transformed with the gene

structure according to the invention, with this value corresponding to an increase by a factor of 18.3 as compared with the non-induced host strain.

5 Example 3

Production of substances using strains which exhibit an increased activity of glucokinase or in which a PEP-independent sugar uptake system is being expressed in addition to the increased activity of the glucokinase

10 The host strain *Escherichia coli* AT2471 and its transformants harbouring one of the plasmids pZY507glk or pZY507glfglk were cultured for 72 h under the standard conditions described in Example 2 in mixtures which were in each case parallel to each other. After 24 and 48 h,
15 the pH of the cultures was measured and, as the need arose, brought back to the starting value of 7.2 by adding KOH (45%). In addition, samples were taken, after 24, 48 and 72 h, for determining the optical density and the concentrations of glucose and L-phenylalanine.

20 The phenylalanine concentrations were measured by means of high pressure liquid chromatography (HPLC, Hewlett Packard, Munich, Germany) in combination with fluorescence detection (extinction 335 nm, emission 570 nm). A Nucleosil-120-8-C18 column (250·4.6 mm) was
25 used as the solid phase; the column was eluted using a gradient (eluent A: 90% 50 mM phosphoric acid, 10% methanol, pH 2.5; eluent B: 20% 50 mM phosphoric acid, 80% methanol, pH 2.5; gradient: 0-8 min 100% A, 8-13 min 0% A, 13-19 min 100% A). The elution rate was set at 1.0 ml·min⁻¹;
30 the column temperature was set at 40°C. Post-column derivatization was carried out using o-phthalic dialdehyde in a reaction capillary (14 m·0.35 mm) at room temperature. L-phenylalanine was found to have a retention time of 6.7 min under the conditions described.

Measurement of the glucose concentration with enzyme test strips (Diabur, Boehringer Mannheim, Germany) and, depending on the results, the subsequent addition of 2 ml of a concentrated glucose solution ($500 \text{ g} \cdot \text{l}^{-1}$) ensured that glucose limitation did not arise in the experimental mixtures. After an incubation time of 48 h, a (phenylalanine) index value of 119 was achieved after inducing the host strain *E. coli* 2471; this compares with a phenylalanine value of 100 for the uninduced host strain. An index value of 141 was achieved for the transformed strain *E. coli* AT2471/pZY507glk simply by increasing the activity of the glucokinase by inducing the strain. By contrast, the introduction of plasmid pZY507glfglk into the host strain enabled phenylalanine production to be increased to a value of 149 even without induction; it was then possible to increase this value still further to 171 by inducing the cells. This corresponded to an increase of 44% as compared with the induced host strain.

This result demonstrates the positive effect according to the invention, which effect increases the synthesis of aromatic compounds, of increasing the activity of a glucokinase in appropriate microorganisms. Furthermore, the result also demonstrates the positive effect exerted on the preparation of the substances by the additional increase in the activity of a glucokinase in strains which already possess an increased presence of a facilitator protein, or by the additional increase in the presence of a facilitator protein in strains which already possess an increased glucokinase activity.

Example 4

Production of substances using PTS⁻ mutants in which a PEP-independent sugar transport system is being expressed

in addition to the increased glucokinase activity

For the purpose of integrating the *glf* gene into genes which encode components of the *E. coli* PTS system, plasmid pPTS1 (see Table 1) was digested with *Bgl*II and treated with Klenow fragment. The unique cleavage site is located in the *ptsI* gene. The *glf* gene was isolated, as a *Bam*HI-*Kpn*I fragment, from plasmid pZY507*glf**glk* and likewise treated with Klenow fragment. Clones carrying the *glf* in the same orientation as the *ptsHI* genes were obtained by blunt-end ligation. A 4.6 kb *Pst*I fragment carrying the 3' region of the *ptsH* gene and *ptsI* containing integrated *glf* and *crr* was obtained from the resulting plasmid pPTS*glf*. This fragment was ligated into the *Eco*RV cleavage site of vector pGP704. Since this vector is only able to replicate in λ pir strains, the vector is integrated into the chromosome in transformants which do not harbour this phage if these transformants are able to grow on carbenicillin (Miller V.L. et al., J. Bacteriol. 170 (1988) 2575-83). The integration was checked by Southern blot analysis. The resulting transformants contained the complete PTS genes in addition to the *glf* gene.

The vector moiety can be recombined out in a second homologous crossover, resulting in the loss of the carbenicillin resistance. Since, in this case, the *pts* genes are interrupted by the insertion of the *glf* gene, the PTS is not expressed in a functional manner in these mutants. The desired PTS⁻ mutants were selected as follows: after subculturing the transformants, which were still PTS⁺, several times on LB medium without antibiotics, aliquots of the cell suspension were plated out on LB plates containing 100 μ g.l⁻¹ phosphomycin. PTS⁻ mutants are able to grow on these plates. Growing clones were streaked out on LB plates containing either phospho-

mycin or $20 \mu\text{g}\cdot\text{l}^{-1}$ carbenicillin. Chromosomal DNA was isolated from clones which still exhibited growth on the phosphomycin plates but which were not able to grow on the carbenicillin plates. The integration of the *glf* gene into the genes which encode the PTS system was confirmed by Southern analysis. Corresponding mutants were identified as being phenotypically PTS-deficient. One clone was selected as the host organism *E. coli* AT2471glfintPTS⁻ and used for the transformations (see above) with plasmid pZY507glk. Following the experimental conditions described for Examples 2 and 3, the PTS-negative mutant *E. coli* AT2471glfintPTS⁻/pZY507glk, and the corresponding host strain AT2471glfintPTS⁻, were in each case cultured in two parallel mixtures and the cells of one mixture in each case were induced after approx. 7 divisions.

The induction reduced the synthetic performance of the host strain, which initially had an index value of 100 without induction, down to a value of 56. By comparison, cultures of the transformed strain AT2471glfintPTS⁻/pZY507glk achieved a phenylalanine index value of 151 even without induction, with this value then being increased by induction to 174.

This result demonstrates that simply increasing the glucokinase activity has a positive effect on the synthesis of phenylalanine even in those microorganisms in which the activity of the PTS system is diminished, or this system is completely switched off, and into which, at the same time, a PEP-independent sugar uptake system has been integrated.

Table 1:

	Strains and plasmids	Genotype/ characteristics	Source or reference
5	<i>E. coli</i> AT2471	<i>tyrA4, relA1, spoT1, thi-1</i>	Taylor and Trotter, Bacteriol. Rev. 13 (1967) 332-53
	<i>E. coli</i> SY327	<i>araD, Δ(lac-pro), Rif^r, recA56, λ-phage pir function</i>	Miller et al., J. Bacteriol. 170 (1988) 2575-83
	<i>E. coli</i> CC118	<i>Δ(ara-leu), araD, ΔlacX74, galE, galK, phoA20, thi-1, rpsE, rpoB, argE (Am), recA1, λpir lysogen</i>	Manoil et al., Proc. Natl. Acad. Sci USA 82 (1985) 8129-33
10	pZY507	Cm ^r	Weisser et al., J. Bacteriol 177 (1995) 3351-4
	pZY507glfglk	<i>Z. mobilis glf and glk genes in pZY507</i>	Weisser et al., J. Bacteriol 177 (1995) 3351-4
	pZY557	pZY507, multiple cloning site of pUCBM20Cm ^r	Sprenger, unpublished

Strains and plasmids	Genotype/ characteristics	Source or reference
pACYC184	Cm ^r , Tet ^r	Chang and Cohen, J. Bacteriol. 134 (1978) 1141-1156
pDIA3206	Ap ^r , 11.5 kb insert from <i>E. coli</i> K12 chromosome including <i>ptsHI-crr</i> genes	DeReuse et al., J. Bacteriol. 170 (1988) 3827-37
pPTS1	pACYC184 containing a 4 kb <i>Cla</i> I fragment which contains the <i>ptsHI</i> and <i>crr</i> genes from pDIA3206; Cm ^r	Jahreis, Osnabruck university, unpublished
pGP704	Amp ^r	Miller et al., J. Bacteriol. 170 (1988) 2575-83

Table 2:

Microorganism	Time after induction/ h	Glucokinase activity/ mU · (mg of protein) ⁻¹	
		without induction	with induction
5 <i>Escherichia coli</i> AT2471	0	21	
	1		
	3	36	
10 <i>Escherichia coli</i> AT2471/pZY507 glfglk	0	23	25
	1	63	202
	3	112	657

C L A I M S

1. Process for the microbial preparation of substances
5 in which the activity of a sugar-phosphorylating
kinase is increased in a microorganism which is
producing these substances.
2. Process according to Claim 1, characterized in that
substances are prepared in whose synthesis phospho-
10 enolpyruvate (PEP) is involved.
3. Process according to Claim 1 or 2, characterized in
that the kinase is a hexose-phosphorylating kinase.
4. Process according to Claim 3, characterized in that
the kinase in the microorganism is derived from
15 *Zymomonas mobilis*.
5. Process according to Claim 4, characterized in that
the kinase is *Zymomonas mobilis* glucokinase (Glk).
6. Process according to one of Claims 1 to 5,
characterized in that the activity of a transport
20 protein for the PEP-independent uptake of a sugar
which is to be phosphorylated by the kinase is
additionally increased.
7. Process according to Claim 6, characterized in that
the activity of a transport protein for the PEP-
25 independent uptake of a relevant sugar is increased
in a substance-producing microorganism which is able
to take up a sugar by means of a PEP-dependent uptake
system.
8. Process according to one of Claims 6 or 7,
30 characterized in that the transport protein is a
facilitator.
9. Process according to Claim 8, characterized in that
the facilitator is the *Zymomonas mobilis* glucose
facilitator protein (Glf).

10. Process according to one of Claims 1 to 9,
characterized in that the activity of a transaldolase
and/or a transketolase is additionally increased.
- 5 11. Process according to Claim 10, characterized in that
the activity of an *Escherichia coli* transaldolase
and/or an *Escherichia coli* transketolase is
increased.
- 10 12. Process according to Claim 10 or 11, characterized in
that the activity of *Escherichia coli* transaldolase B
(TalB) and/or *Escherichia coli* transketolase A (TktA)
is increased.
- 15 13. Process according to one of Claims 1 to 12,
characterized in that the activity of the PEP-
dependent sugar uptake system, if present, is
decreased as compared with the natural level.
- 20 14. Process according to one of Claims 1 to 13,
characterized in that the activity of at least one of
the components sugar-phosphorylating kinase,
transport protein, transaldolase and transketolase is
increased
- 25 a) by introduction of the genes
b) and/or by increasing the gene copy number
c) and/or by increasing gene expression
d) and/or by increasing the endogenous activity of
the said enzymes
e) and/or by altering the structure of the enzymes
f) and/or by using deregulated enzymes
g) and/or by inserting genes which encode
deregulated enzymes.
- 30 15. Process according to Claim 14, characterized in that
the increase in activity is achieved by integrating
the gene or the genes into a gene structure or into
several gene structures, with the gene or the genes
being introduced into the gene structure as (a)

- single copy(ies) or at an elevated copy number.
16. Process according to one of Claims 1 to 15,
characterized in that a microorganism is employed in
which one or more enzymes, which are additionally
involved in the synthesis of the substances, are
deregulated and/or have an increased activity.
17. Process according to Claim 16, characterized in that
the substance which is prepared is an aromatic amino
acid.
18. Process according to Claim 17, characterized in that
the aromatic amino acid is L-phenylalanine.
19. Process according to one of Claims 1 to 18,
characterized in that the microorganism employed
belongs to the genus *Escherichia*, *Serratia*, *Bacillus*,
Corynebacterium or *Brevibacterium*.
20. Process according to Claim 19, characterized in that
the microorganism is *Escherichia coli*.
21. Gene structure containing, in recombinant form, a
gene encoding a sugar-phosphorylating kinase and a
gene for a transport protein for the PEP-independent
uptake of a sugar, with the exception of a
combination of the genes *glk*, for the *Zymomonas*
mobilis kinase, and *glf*, for the *Zymomonas mobilis*
transport protein.
22. Gene structure containing, in recombinant form,
a) a gene encoding a sugar-phosphorylating kinase
or genes encoding a sugar-phosphorylating kinase
and a transport protein for the PEP-independent
uptake of a relevant sugar, and
b) at least one gene encoding a transaldolase or a
transketolase.
23. Gene structure according to Claim 21 or 22,
characterized in that the gene for the sugar-
phosphorylating kinase encodes a hexose-

phosphorylating kinase and the gene for the transport protein encodes a facilitator.

24. Gene structure according to one of Claims 21, and/or 22 or 23, characterized in that the genes for the kinase and for the transport protein are derived from *Zymomonas mobilis* and in that the genes for the transaldolase and the transketolase are derived from *Escherichia coli*.
25. Gene structure according to one of Claims 22 to 24, characterized in that the gene for the *Zymomonas mobilis* kinase is *glk* and the gene for the *Zymomonas mobilis* transport protein is *glf* and the gene for the *Escherichia coli* transaldolase is *talB* and the gene for the *Escherichia coli* transketolase is *tktA*.
26. Gene structure according to one of Claims 22 to 25, characterized in that the *glf* gene is inserted into the gene structure at low gene copy number.
27. Gene structure according to one of Claims 21 to 26, characterized in that the gene structure contains at least one regulatory gene sequence which is assigned to one of the genes.
28. Gene structure according to one of Claims 21 to 27, characterized in that at least one of the genes in the gene structure is incorporated such that it is under the control of an inducible promoter.
29. Gene structure according to Claims 22 to 28, characterized in that at least two of the genes are under the control of two opposed promoters.
30. Transformed cell, harbouring, in replicatable form, at least one gene structure according to Claims 21 to 29.
31. Transformed cell according to Claim 30, characterized in that one or more enzymes, which are additionally involved in the synthesis of the substances, are

deregulated and/or have an increased activity.

32. Transformed cell according to Claim 30 or 31, characterized in that the cell is an *Escherichia coli* cell.
- 5 33. Transformed cell according to one of Claims 30 to 32, characterized in that the PEP-dependent sugar uptake system, if present, has a reduced activity as compared with the natural level or is abolished.
- 10 34. Transformed cell according to one of Claims 30 to 33, characterized in that it is able to produce an aromatic amino acid.
35. Transformed cell according to Claim 34, characterized in that the aromatic amino acid is L-phenylalanine.
- 15 36. Process according to one of Claims 1 to 20, characterized in that transformed cells according to one of Claims 30 to 35, in which there is a gene structure according to Claim 28 or 29, are cultured, with the induction being effected after two cell divisions at the earliest.
- 20 37. Process according to Claim 36, characterized in that, in addition to PEP, the transformed cell also contains other metabolites of central metabolism in increased availability.

INTERNATIONAL SEARCH REPORT

Inter national Application No

PCT/NL 97/00583

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/54 C12P13/22 C12P1/00 C12P7/42 C12N15/31
C12N1/21 //C12Q1/48, (C12N1/21, C12R1:07, 1:13, 1:15, 1:185, 1:19,
1:425)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12P C07K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FROST J.W. AND DRATHS K.M.: "Biocatalytic syntheses of aromatics from D-glucose: renewable microbial sources of aromatic compounds" ANNUAL REVIEW OF MICROBIOLOGY, vol. 49, 1995, pages 557-579, XP002053641 cited in the application	1-20, 22-37
Y	see page 559, paragraph 2 - page 564 --- -/--	10-12, 21



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

5 February 1998

Date of mailing of the international search report

20/02/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Macchia, G

INTERNATIONAL SEARCH REPORT

Inter nat Application No
PCT/NL 97/00583

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SPRENGER G.A. ET AL.: "Transaldolase B of Escherichia coli K-12: cloning of its gene, talB, and characterization of the enzyme from recombinant strains" JOURNAL OF BACTERIOLOGY, vol. 177, no. 20, October 1995, pages 5930-5936, XP002053305 cited in the application see page 5930, left-hand column, line 1-18 see page 5934, left-hand column; table 2 see page 5935, right-hand column, line 8-21 ---	10-12
Y	FLORES N. ET AL.: "Pathway engineering for the production of aromatic compounds in Escherichia coli" NATURE BIOTECHNOLOGY, vol. 14, May 1996, pages 620-623, XP002053707 cited in the application see page 620 ---	21
A	BERRY A.: "Improving production of aromatic compounds in Escherichia coli by metabolic engineering" TRENDS IN BIOTECHNOLOGY, vol. 14, no. 7, July 1996, page 250-256 XP004035764 see page 254, right-hand column, paragraph 3 - page 255, left-hand column, paragraph 1 ---	1-3,6-8, 13
X	WEISSER P. ET AL.: "Functional expression of the glucose transporter of Zymomonas mobilis leads to restoration of glucose and fructose uptake in Escherichia coli mutants and provides evidence for its facilitator action" JOURNAL OF BACTERIOLOGY, vol. 177, no. 11, June 1995, pages 3351-3354, XP002053706 cited in the application see page 3351 ---	1-3,6, 10-20
Y	ZHANG M. ET AL.: "METABOLIC ENGINEERING OF A PENTOSE METABOLISM PATHWAY IN ETHANOLOGENIC ZYMOMONAS MOBILIS" SCIENCE, vol. 267, 13 January 1995, pages 240-243, XP002026045 see page 241 ---	4,5,7-9, 22-37
Y		4,5,7-9, 22-37
X		1,2, 10-16, 22, 27-31, 33,36

-/--

INTERNATIONAL SEARCH REPORT

Inter. nat Application No

PCT/NL 97/00583

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	US 5 602 030 A (INGRAHM LONNIE O ET AL) 11 February 1997 see the whole document ---	1-6,8,9, 13-16, 19,20
A	PARKER C. ET AL.: "Characterization of the Zymomonas mobilis glucose facilitator gene product (glf) in recombinant Escherichia coli: examination of transport mechanism, kinetics and the role of glucokinase in glucose transport" MOLECULAR MICROBIOLOGY, vol. 15, no. 5, March 1995, pages 795-802, XP002054312 cited in the application ---	
A	GOSSET G. ET AL.: "A direct comparison of approaches for increasing carbon flow to aromatic biosynthesis in Escherichia coli" JOURNAL OF INDUSTRIAL MICROBIOLOGY, vol. 17, no. 1, July 1996, pages 47-52, XP002054313 see abstract see page 50; table 2 -----	

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/NL 97/00583

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5602030 A	11-02-97	NONE	